Investigating the impacts of soil quality on floral color and pollinator perception

Undergraduate Research Thesis

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by

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1. **Abstract**

Prairies are vital ecosystems that have declined to 1% of their original landcover in the United States over the past 150 years. To conserve these endangered habitats, practitioners have established prairies on land previously used for surfacing mining. The soil quality of these prairies varies widely depending on how the land was reclaimed and revegetated following mining. Differences in soil quality can alter plants' physical traits, but little is known about the impacts of soil quality on plant-pollinator interactions. The objective of this study was to determine if reclamation technique affects the floral optics (reflectance, luminance, pattern) and pollinator perception of floral resources. We hypothesized that how a prairie was reclaimed, as represented by its reclamation permit, affects the optical properties of flowers and their appearance to pollinators. We photographed the standard and wing petals of Bird's Foot Trefoil (*Lotus corniculatus*), an important pollinator resource frequently used in each reclaimed prairie site. Sites were located at The Wilds, a conservation center established on reclaimed surface mine lands that span several different reclamation permits. We took photos of 20 flowers from each site in a controlled environment using a UV/IR sensitive camera that collected wavelengths in both the visible and ultraviolet spectrums. We then analyzed the photos using multispectral image calibration software to measure reflectance, luminance, and pattern. Across sites with different reclamation permits, reflectance values of visible blue on standard and wing petals were significantly different. Ultraviolet blue, ultraviolet red, cone-catch ultraviolet, and anthocyanin concentrations differed significantly between permit type and flower part. These differences suggest that bees may view flowers differently across reclamation permits, which could affect their likelihood to visit those resources.

Key words: Bee, calibrated photographs, cone-catch images, conservation, multispectral images, prairie, reclamation, The Wilds

2. **Introduction**

Prairies provide vital ecosystem services. They prevent erosion, sequester carbon, and maintain biodiversity (Breckenridge *et al.* 2008). Ecologically functional prairies also provide habitat for a wide range of pollinators (Gilgert & Vaughan 2011). The diverse flowering plants, bare ground, and dead wood present in prairies support rapidly declining native bee populations (Russell *et al.* 2005). Habitat protection for bees is critical as they are responsible for the pollination of most flowering plants and 66% of the world's crop species (Russell *et al.* 2005).

Over the past 150 years, habitat loss has decimated prairies. They are now considered one of North America's most endangered ecosystems (Samson *et al.* 2010). The conversion of grassland to cropland often results in desertification, the processes by which previously healthy soil becomes dry and barren. The conversion to agriculture has damaged their associated ecological communities, including pollinator populations (Ceballos 2010). A promising source of new prairies stems from land previously used for surface coal mines.

Nearly 1.5 million acres have been mined in the Appalachian region (USGS 2011). Much of this land was originally forested. As a condition of mining, companies must reclaim land after mining is complete. The reclamation requirements for surface mining have evolved over the last 70 years, with the earliest requirements having the lowest standards. Early laws did not require the same level of revegetation outlined by more recent laws (Table 1). Today, the regrading of land and replacement of topsoil followed by the revegetation of appropriate plant species are required when reclaiming surface-mined land (Skousen 2014). Because regulations do not specify a revegetation type, many of these previously forested sites are reclaimed as grasslands. This method is quicker, easier, and more cost-effective than forest reclamation (Swab *et al.* 2017).

Recent interest has increased in establishing prairie species on reclaimed mine land. The Wilds, a nearly ten-thousand-acre conservation park of the Columbus Zoo, is one of the largest areas of recovering surface mine land in North America, with almost 700 acres revegetated as prairie habitat (Cavendar *et al.* 2014). At the Wilds, there are at least four different classes of land reclamation, distinguished by the permits that directed reclamation techniques. A Permit sites (1966-73) are the oldest and experienced no soil improvements. B Permit sites (1973-1976) were leveled and reseeded with cool-season grasses. The youngest sites with the strictest regulations, C and D Permit sites (1975-2002), were reseeded and replanted following mining activities, as directed by the Surface Mining Control and Reclamation Act of 1977 (SMCRA) (Zook *et al.* 1990). SMCRA was formed after older mining reclamation laws failed to show visible progress in improving land (Zook *et al.* 1990).

Land that has been surface mined and subsequently reclaimed can vary widely in soil quality, which can in turn impact prairie establishment. Prairie establishment is crucial to create habitat for pollinators, and pollination is crucial for reproductive success of both plants and pollinators, and for the ultimate success of prairie restoration. The surface mining process destroys all life above ground while disrupting soil below ground (USDI 1979). When topsoil is removed, the soil microbial populations are altered (Waaland & Allen 1987). Mining activity can also reduce soil organic carbon and nitrogen concentrations while increasing pH (Shrestha 2011). Mining may also contaminate soils with heavy metals (Weissmannova *et al.* 2017), the severity of which likely depends on reclamation technique. Despite this, newly established prairies on reclaimed mine land have demonstrated improvements in their soil, such as better microbial communities (Swab *et al.* 2017). These communities help increase nutrient availability and reduce possible erosion (Shetty 1993). Prairie success is also likely influenced by the extent of reclamation employed; a factor dependent on the regulations in place at the time that the mining permit was issued (Zook *et al.* 1990). Preliminary data collected from soil samples at the Wilds suggests that the soils in different permit classes vary in quality. Older B Permit sites with less reclamation requirements have higher heavy metal contamination than the younger C Permit sites (Fig. 1; *F. Sivakoff unpublished*). Heavy metal contamination in B and C sites were estimated with pollution load index scores that consider all heavy metal concentrations and background concentration levels in the soil (Weissmannova & Pavlovsky 2017). Heavy metal content in A Permit sites is currently awaiting analysis.

Heavy metals found in reclaimed mine land have the potential to bioaccumulate in native plant species (Nawab 2015), yet the effects of soil quality on plant-pollinator interactions are largely unexplored. These accumulations may affect the flower characteristics that are displayed to pollinators, such as pigmentation (Meindl *et al.* 2013; Baek *et al.* 2012). For example, *Arabidopsis* flowers grown in soil contaminated with Cu, Hg, Mn, Pb, or Zn can exhibit significantly lower levels of chlorophyll and significantly higher levels of carotenoids and anthocyanin (Baek *et al.* 2012). Heavy metal contamination in soils can also affect pollinator foraging and visitation time on some flowering plant species, indicating that plant-pollinator interactions may be influenced under poor soil conditions (Meindl & Ashman 2014; Sivakoff & Gardiner 2017). Pollinators are attracted to a variety of floral traits, such as color, aroma, and nectar quantity (Murrell 1982). Bees are attracted to distinctive color signals and nectar guides on flowers that appear in short-distance orientations (Dyer 2003; Lunau 1996; Murrel 1982). Given this, alterations in color that result from differences in soil quality could have major consequences for plant-pollinator interactions and for the success of prairie restoration.

Unlike humans, bees are trichromatic and see the colors reflected by a flower in the visible and ultraviolet wavelengths (Dyer *et al.* 2011; Srinivasan 2010). Because of the visual system inconsistency, measuring color differences relevant to bee vision with a human eye is misleading. Previously, spectrometry has been the favored technique for taking objective measurements of color (Stevens *et al.* 2007; Troscianko & Stevens 2015). In this process, spectrophotometers are used to collect information on the distribution of reflected wavelengths (Stevens *et al.* 2007). However, this method is both costly and time consuming. Instead, digital photography and image processing are accessible and reliable methods for measuring color in the visible and ultraviolet spectrums (Valle *et al.* 2018). Images captured with modified multispectral cameras (cameras that can detect infrared to ultraviolet wavelengths) can be normalized into multispectral images to control for lighting conditions (Troscianko & Stevens 2015). Objective measurements of color reflectance can then be gathered from multispectral images. Multispectral images can then be converted to model the visual systems of animals, such as the honey bee, using known spectral sensitivities (Troscianko & Stevens 2015).

In this study, we investigated how the reclamation permit and soil quality of reclaimed mine land can affect floral optics (reflectance, luminance, and pattern) and pollinator perception of floral resources by using a modified UV camera to photograph flowers grown in different permit areas of the Wilds. We hypothesized that flowers present in sites with differing reclamation permits would vary in their optical properties and appearance to pollinators.

3. **Methods**

a. Study system

We conducted fieldwork in July and August of 2019 at the Wilds, a 10,000-acre conservation park in Eastern Ohio. There are four different classes of land reclamation at the Wilds, distinguished by the permits that directed reclamation techniques (Table 1; Zook *et al.* 1990). We selected 11 reclaimed prairie sites, three were in A permit areas, four in B permit, three in C permit, and one in D permit (Fig. 2). Specific sites were selected based on their permit type, prairie qualities, and accessibility.

b. Flower sampling

Lotus corniculatus (Bird's foot trefoil) is a flowering legume that is widely distributed across prairies at the Wilds. The flower is an important resource for pollinators (Murrell 1982) and is readily able to withstand the abiotic stresses associated with reclamation mining (Escaray 2012). A study conducted by Nawab *et al.* (2015) found that *L. corniculatus* can bioaccumulate the same heavy metals which are found at the Wilds. We sampled *L. corniculatus* by collecting 20 freshly opened flowers at each site. To ensure that all flowers were freshly opened, we identified and bagged these flowers one day prior to collection. Once collected, we placed the flowers on ice to preserve their color and transported them to the photo studio. We dissected, arranged, and photographed each flower on the same day that they were collected. The standard petal and one wing petal from each flower were dissected for photographs. Papilionaceous flowers, like *L. corniculatus*, have five petals: one standard petal, two wing petals and two keel petals (Fig. 3). We haphazardly chose which wing petal to photograph, resulting in a roughly equal number of left- and right-wing petals photographed.

c. Flower photography

i. Studio

We created a controlled studio environment with a UV light source, diffuser, scale bar, and color standard that allowed for consistency between standardized photographs, as suggested in methods developed by Troscianko and Stevens (2015) (Fig. 4 & 5). No light sources were present in the room other than the UV light source, a 50-watt metal halide bulb that provided visible light, heat and UVA and UVB (SunRay PT2327 - 50W, Exo Terra Hagen Corporation, Mansfield MA), positioned 34.5 cm above and 25 cm away from the 9 cm by 13 cm frame. The light illuminance was approximately 32700 LUX with 4500 $\mu W/cm^2$ UVA and 255 $\mu W/cm^2$ UVB. We placed a Teflon sheet 14 cm away from the frame between the light source to eliminate shadows.

ii. Camera

We used the Fuji FinePix S3 Pro to photograph the collected flowers. The camera was modified to capture light on the infrared, visible, and ultraviolet spectrums and was equipped with a Coastal Optical 60 mm 1:4 UV-VIS-IR Apo Macro lens (Jenoptik, Jupiter, FL, USA) attached to a F-mount (Nikon, Melville, NY, USA). First, we photographed each set of flowers on the visible spectrum with the Baader UV IR Cut Luminance Filter 2 Eyepiece Thread (Baadar Planetarium, Mammendorf, Germany). We then photographed the flowers on the ultraviolet spectrum with the Baadar U-Venus Filter 2 (Baadar Planetarium, Mammendorf, Germany). For all photos, camera settings were as follows: Aperture 4.0; ISO 200; Shutter Speed 1/2000-30.0. Shutter speeds varied due to bracketing all visual and ultraviolet photos to achieve correct exposure. All photos were saved in the RAF format, Fuji's version of RAW.

d. Image capture

To photograph a site's 20 flowers, we randomly chose half of the flowers and arranged their standard and wing petals on a white Teflon sheet within a 9cm by 13cm area that made up the photo frame. A 12 cm scale bar and a 99% white PTFE tape standard and 18% grey standard were also placed in the photo to allow for later processing between photos (Fig. 6).Each site had two photo frames arranged to account for all 20 flowers. All photo frames were then analyzed, other than one frame from one B Permit site that was discarded due to processing issues.

e. Photo analysis

Photos were analyzed using the ImageJ (Schneider *et al.* 2012) micaToolbox V2 plugin 7 (Troscianko & Stevens 2015). This free ImageJ plugin was developed by sensory ecologists to objectively measure reflectance, color, and pattern by combing light channels from multispectral cameras, such as the modified camera being used in this experiment (Troscianko & Stevens 2015). So far, the method has been utilized in research regarding animal body color and camouflage (Ospina *et al.* 2017). Measured light properties can also help estimate pollinator color perception by allowing the adjustment of photographs to wavelengths normally invisible to humans (Johnsen 2016).

We first used the micaToolbox to convert the images into calibrated multispectral images. This is necessary to make objective measurements because the tool "normalizes" the image, controlling for differences in lighting, color, and exposure (Troscianko & Stevens 2015). The toolbox selected bracketed images for each site with the best exposures based on their color histograms. The corresponding visible and UV images for a site were then inputted, resulting in a linear color image output. We measured the average Red, Blue, Green, UV Red, UV Blue, and

UV Green reflectance values of the standard and wing of each flower. This was done utilizing the ROI, tracing, and measurement tools of ImageJ and the micaToolbox (Fig. 7A, B, & C).

Using the known visual system of the European Honey Bee (*Apis mellifera*) (Dyer *et al.* 2011), we converted these multispectral images into cone-catch images (Fig. 7D). Cone-catch image conversion produces photos that are based on a given visual system, independent of the camera used (Troscianko & Stevens 2015). With this, we can create false-color images that show the reflectance a honey bee may see when looking at the same image. The cone-catch model used was generated using a spectral-sensitivity-based model. This required the input of spectral sensitivities for the Fuji FinePix s3 Pro (Hunt et al. 2010; Garcia et al. 2014). The cone-catch conversion also required the known spectral sensitivities of the honey bee visual system, which were pre-loaded into the micaToolbox and crossed checked with Dyer *et al.* (2011) and Vorobyev (2001). We then measured the average UV wave, short wave, and medium wave of the standard and wing for each flower. To create false-color images (Fig. 7D) from the cone-catch models, we transformed the UV, short, and medium wavelengths to visible blue, green, and red.

f. Data analysis

We used the statistical software R (R Core Team 2013) to perform an analysis of variance across all reflectance measurements of standard and wing to evaluate their relationship with permit type. The reflectance measurements that we evaluated were: Visible Red, Visible Green, Visible Blue, Ultraviolet Red, Ultraviolet Blue, Cone-catch Short Wave, Cone-catch Medium Wave, Cone-catch Ultraviolet, Luminance, and Cone-catch Luminance. Reflectance values were then used to calculate Hue, Chroma, Brightness, and Anthocyanin concentrations. These values were calculated with equations from del Valle *et al.* (2017):

$$\textbf{Hue}: H = (2 * r - g - b)/(g - b)$$

$$\textbf{Brightness}: B=\sqrt{[(b^2 + g^2 + r^2)/3]}$$

$$\textbf{Chroma}: C = (N_{red} - N_{green})/((N_{red} + N_{green} + N_{blue})/3)$$

$$\textbf{Anthocyanin Content}: AC_{CB} = (N_{blue} + N_{red})/N_{green}$$

Each response variable was modeled separately, and all models included flower part (standard or wing petal) and whether the petals were photographed in the first or second photo as fixed effects. Also included as fixed effects were permit type and the interaction between permit type and flower part. Site was included as a random effect. When we saw a significant difference between permit types, we performed a post hoc tukey test to determine what differences were present in reflectance between sites. UV blue, UV red, UV cone-catch, UV short-wave, and cone-catch luminance were log transformed prior to analysis to meet assumptions of normality.

We created honey bee color space plots to model the color of standard wing and petals in R using medium, short, and UV wavelengths measured from the cone-catch models. Dots represent average color at a permit site. Colored ellipses represent different permit type, with the closer two ellipses are to each other, the more similar the groups are to each other. The color space plots allow for the determination of color differences perceived by honey bees.

4. **Results**

Visible Blue reflectance values were significantly different between reclamation permits B and C ($p = 0.025$), with B permits having higher values than C permits (Table 2; Fig. 8). Flowers from A Permit sites had intermediate values and were not significantly different from B (p =0.333) nor C (p =0.289). This trend is true for standard and wing petals. Visible Red and Visible Green values were not significantly different between permit types ($p > 0.05$; Table 2).

Ultraviolet Red and Ultraviolet Blue values were also significantly different between permit types B and C, but not permit type A (Table 2; Fig. 9 & 10). In both cases, B Permit had higher reflectance values (Table 5). This relationship differed for wing and standard petals (interaction between flower part and permit; UV Blue: $p < 0.001$, UV Red: $p = 0.003$). For both UV Blue and UV Red values, the difference between B and C Permits for the standard petal was larger than for the wing petal (UV Blue: $p = 0.006$ vs. $p = 0.012$; UV Red: $p = 0.008$ vs. $p = 0.014$).

Measured luminance values were not significantly different between permit sites. Calculated hue, brightness, and chroma values were also not significantly different between permit sites (Table 3 & 4). However, calculated anthocyanin values were significantly different between permit types B and C (Table 6; Fig. 11). This relationship differed for wing and standard petals (Interaction between flower part and permit: $p < 0.001$), with a greater difference between B and C permits for standard petals compared to that of the wings ($p = 0.03$, $p = 0.034$).

Cone-catch ultraviolet reflectance values were also significantly different between permit types B and C (Table 3; Figure 12). This relationship differed for wing and standard petals (Table 4), with a greater difference between B and C permits for standard than for wing ($p = 0.012$, $p = 0.039$). Cone-catch UV reflectance values were mapped with cone-catch medium and short wavelength reflectance to values to create a honey bee space color plot (Fig. 13). The plot suggests that standard petals grown in B and C sites may appear different to honey bees. This relationship has not been formally evaluated.

5. **Discussion**

Lotus corniculatus grown in different permit areas reflect significantly different amounts of visible blue, ultraviolet blue, and ultraviolet red wavelengths. But for some measurements, significant differences depended on the flower part. These reflectance differences may affect what pollinators see when searching for floral resources. Honey bee (*Apis mellifera)* color space plots (Fig. 13) suggest that bees may perceive differences between standard petals from B and C sites, but not wing petals. Perceived differences may be due to the nectar guides, markings that attract pollinators to the nectar in a flower, in *L. corniculatus* being present only on the standard petal (Murrel 1981). The differences between nectar guides on standard and wing petals of *L. corniculatus* could be responsible for the differences in interactions between flower part and permit type (Table 5 & 6). While we know that bees view standard petals differently between B and C permit sites, we do not know if this difference translates into different preferences or visitation rates. In the future, we could measure pollinator visitation rates at *L. corniculatus* to see if these differences translate into different preferences. It is also worth noting that because bees are trichromates, they see can only see visible blue, visible green, and the UV spectrum (Dyer *et al.* 2011), indicating that any differences between visible red reflectance values, while non-significant, would not appear to bees. Luminance, brightness, hue, and chroma were also not different between permit types. These values can be indicative of differences in pigmentation concentrations and patterns (de Valle *et al.* 2017). Anthocyanin concentrations were also different between both flower part and permit type. However, anthocyanin concentrations have only been measured in the stem and leaves of *L. corniculatus* in the past, so their average flower petal concentrations are unknown (Robbins *et al.* 2003).

Color is an important attractant to pollinators when searching for resources (Dyer 2003; Lunau 1996; Murrel 1982). However, nectar production in *L. corniculatus* is not strongly linked to petal color (Murrell *et al.* 1982). Therefore, color may not be a strong indicator to pollinators distinguishing specifically between *L. corniculatus* flowers. Additionally, while *L. corniculatus* has been confirmed to accumulate heavy metals when grown in contaminated soils (Nawab *et al.* 2015), it is uncertain how flower color may be affected by heavy metal accumulation in the plant, or if the accumulation of heavy metals could interact with color reflectance properties. A better understanding of the chemical interactions inside *L. corniculatus* may clarify the linkage between metal accumulation and floral trait display.

While our results suggest that soil quality is important for floral color, we cannot disentangle the effect of soil from other environmental factors. Sunlight, canopy cover, and competing foliage may have interacted with the growth of *L. corniculatus*, impacting its ability to reflect color. Soil characteristics other than heavy metal concentration, such as compaction, organic carbon, or pH, may also contribute to reflectance differences. To control for these relationships, the effects of soil quality on floral traits must be isolated, which is planned as future work.

Limitations also arose in image processing. When converting RAF images to multispectral images, the second photo frame for site ZZO could not be processed for an undetermined reason. This resulted in this site only having measurements from 10 flowers, rather than 20 and being excluded from further analyses. Additionally, ultraviolet and short-wave reflectance values in cone-catch images came back mostly negative. These results should be treated with caution because these colors are most like beyond the limit of the cone-mapping model's color reproduction abilities (Troscianko & Stevens 2015).

6. **Conclusion**

This project explored how soil quality can alter floral characteristics and what pollinators see when they search for resources. We tested the hypothesis that plants grown in different degrees of stressed soil, as dictated by reclamation permit type, reflect color differently and thus are viewed differently by pollinators. Reclamation methodology, as illustrated by permit type, results in soil quality differences and contributes to differences in floral traits. However, it is unclear whether only soil quality can account for these differences. Going forward, it is necessary to isolate the effects of soil quality on floral traits to address these uncertainties. Additionally, neither *Lotus corniculatus* nor *Apis millifera* are native species to the Appalachian region. Utilizing species that are native to the Appalachian region to investigate plant and pollinator interactions on reclaimed mine land can inform native restoration techniques. Pollination is crucial for the reproductive success of both plants and pollinators, and for the ultimate success of prairie restoration. Investigating the impacts of reclamation permits on floral traits can aid in the understanding of prairie ecological systems and inform future prairie restoration efforts.

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9. **Tables and Figures**

Table 1: Ohio Mining Reclamation Permits. Table modified from Zook *et al*. 1990.

Permit Type	Years Affected	Regulation Name	Permit Regulation
A	1966-73	Ohio Coal Strip Mine Land Reclamation Act (1949)	Regrading, some reclamation planning and replanting
B	1973-76	Ohio Strip Mine Law (1972)	Regrading and establishment of cool season grasses
C-D	1975-02	Surface Mining Control and Reclamation Act (SMCRA) (1977)	Strict regrading and revegetation that resembles landscape before mining

Table 2: Interaction of visible red, visible green, visible blue, ultraviolet red, and ultraviolet blue with the fixed effects of permit type, flower part, picture, and permit with flower part.

	V. Red		**V. Green**		**V. Blue**		**UV Red**		**UV Blue**	
Fixed Effects	**z**	***p***	**z**	***p***	**z**	***p***	**z**	***p***	**z**	***p***
Permit type	1.71	0.425	1.58	0.453	11.2	0.004	15.4	< 0.001	15.3	< 0.001
Flower part	33.5	< 0.001	13.9	< 0.001	212	< 0.001	541	< 0.001	5890	< 0.001
Picture	6.22	0.013	9.48	0.002	6.26	0.012	0.045	0.832	0.313	0.576
Permit x flower part	7.47	0.024	8.44	0.015	0.156	0.925	11.7	0.003	15.2	< 0.001

Table 3: Interaction of cone-catch medium wave, cone-catch ultraviolet wave, hue, and chroma with the fixed effects of permit type, flower part, picture, and permit with flower part.

	C.C. Medium Wave		**C.C. UV Wave**		**Hue**		**Chroma**	
Fixed Effects	**z**	***p***	**z**	***p***	**z**	***p***	**z**	***p***
Permit type	3.69	0.158	12.2	0.002	4.17	0.124	1.21	0.547
Flower part	5.46	0.019	465	< 0.001	3.09	0.087	48.6	< 0.001
Picture	11.3	< 0.001	0.067	0.795	3.75	0.053	4.2	0.040
Permit x flower part	8.24	0.016	9.84	0.007	10.7	0.004	6.22	0.044

Table 4: Interaction of brightness, anthocyanin concentration, multispectral luminance, and cone-catch luminance with the fixed effects of permit type, flower part, picture, and permit with flower part.

	Brightness		**Anthocyanin**		**Multispectral Luminance**		**Cone-catch luminance**	
Fixed Effects	**z**	***p***	**z**	***p***	**z**	***p***	**z**	***p***
Permit type	2.229	0.328	10.3	0.006	8.95	0.011	11.9	0.002
Flower part	19.2	< 0.001	1890	< 0.001	107	< 0.001	1850	< 0.001
Picture	7.91	0.005	0.356	0.551	3.26	0.071	0.011	0.915
Permit x flower part	8.5	0.014	14.2	< 0.001	0.364	0.834	4.64	0.098

Table 5: Mean reflectance values of UV Red and UV Blue from the standard and wing petals at A, B, and C sites.

	UV Red				UV Blue			
Flower part	**Permit Type**	**Mean**	**- CI**	**+ CI**	**Permit Type**	**Mean**	**- CI**	**+ CI**
Standard	A	2.6	2.5	2.7	A	2.44	2.31	2.56
	B	2.72	2.62	2.81	B	2.59	2.46	2.71
	C	2.5	2.42	2.57	C	2.29	2.19	2.38
Wing	A	2.75	2.66	2.85	A	2.63	2.51	2.76
	B	2.91	2.82	3.01	B	2.82	2.69	2.95
	C	2.72	2.65	2.79	C	2.58	2.48	2.67

Table 6: Mean reflectance values of UV Red and UV Blue from the standard and wing petals at A, B, and C sites.

	Cone-catch UV				Anthocyanin			
Flower part	**Permit Type**	**Mean**	**- CI**	**+ CI**	**Permit Type**	**Mean**	**- CI**	**+ CI**
Standard	A	-2.23	-2.37	-2.09	A	1.43	1.37	1.48
	B	-2.09	-2.24	-1.95	B	1.46	1.4	1.52
	C	-2.04	-2.51	-2.29	C	1.36	1.31	1.4
Wing	A	-2.01	-2.15	-1.86	A	1.45	1.39	1.51
	B	-1.85	-1.99	-1.71	B	1.51	1.45	1.57
	C	-2.09	-2.2	-1.98	C	1.41	1.37	1.46

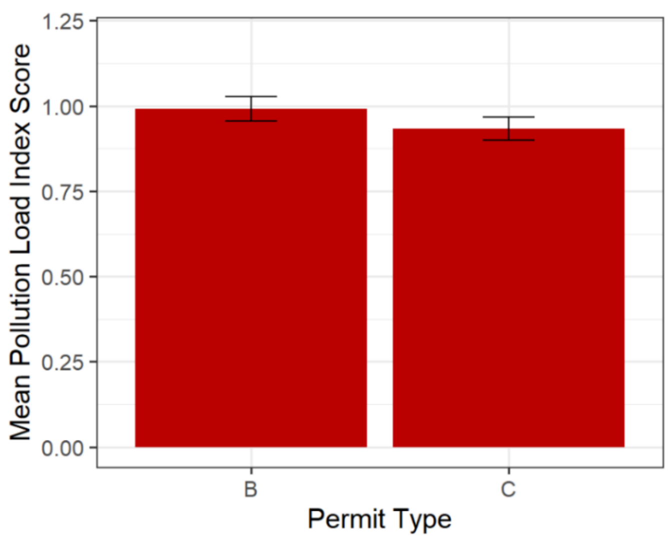

Figure 1: Pollution load index (PLI), a measure of soil quality where higher values suggest poorer quality, is trending higher in older B Permit sites than younger C Permit sites (P=0.07; F. Sivakoff, unpublished). PLI scores take into account concentrations of 18 different metals in the soil. A Permit sites were collected later and are currently being analyzed.

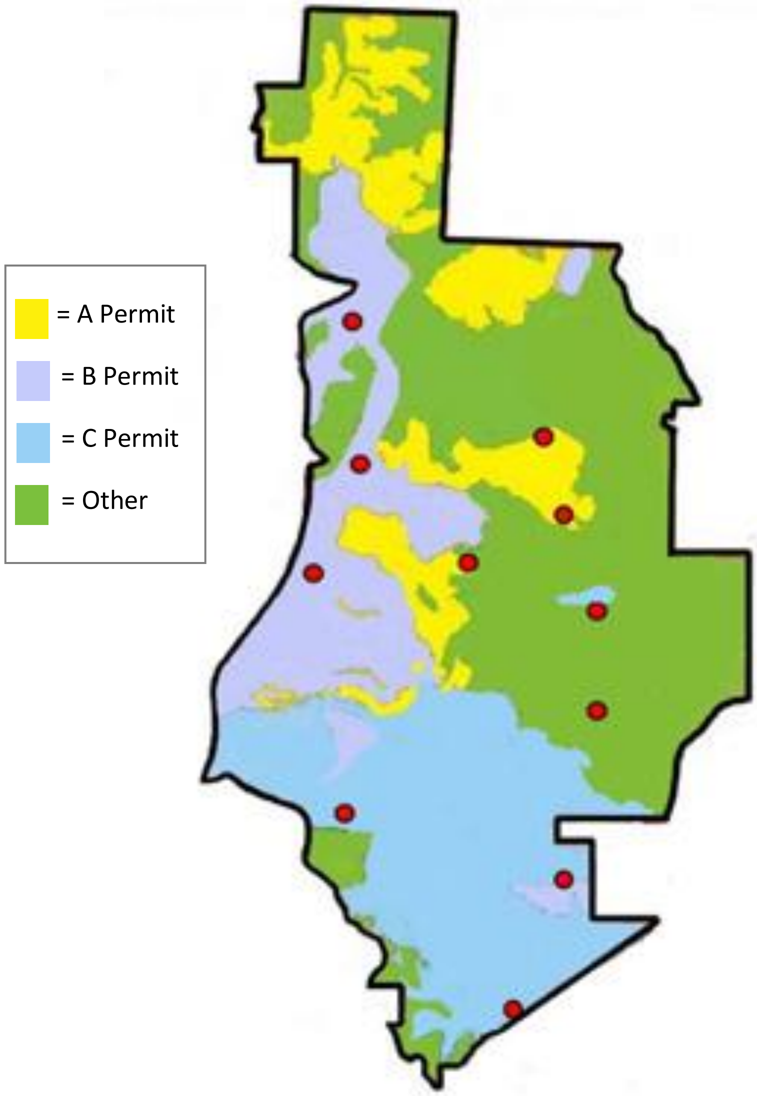

Figure 2: Map of the Wilds highlighting previously mined areas and remnant forest. Different permit classes (A-C) indicate different periods of mining and different reclamation methodology. Red dots indicate the 11 sites sampled for this study. Modified from the Wilds (*unpublished*).

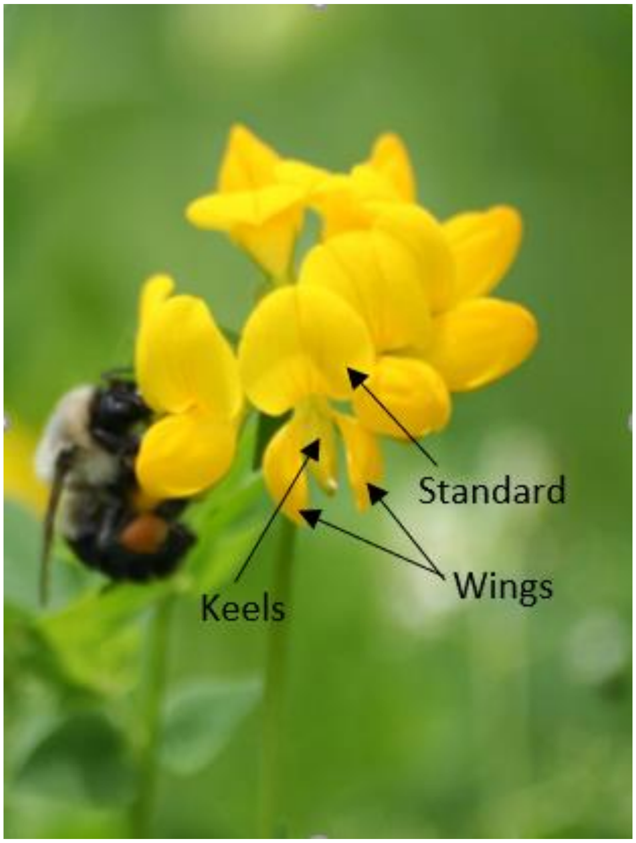

Figure 3: Standard, wing, and keel petals on *Lotus Corniculatus.* Photo by John Ballas.

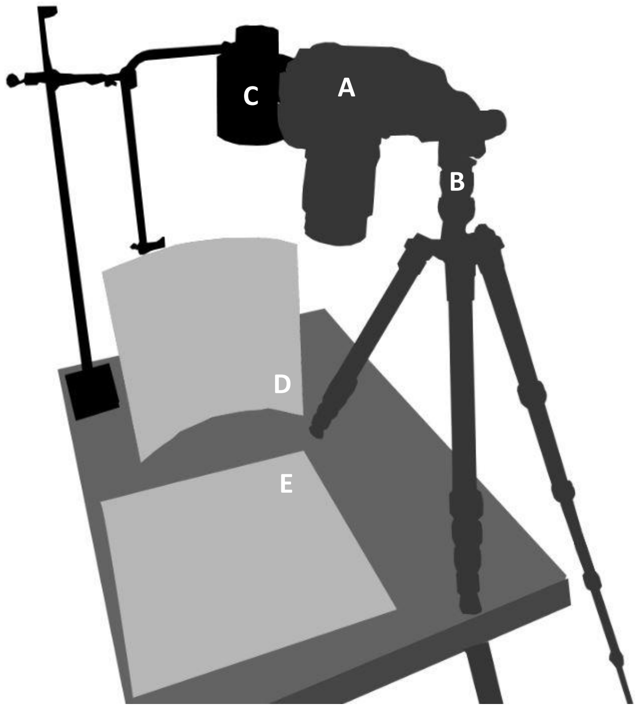

Figure 4: A side view of the studio set up for photographing *L. corniculatus.* The (A) camera, (B) tripod, () light source, and (D & E) teflon sheets were used to create a dark room setting.

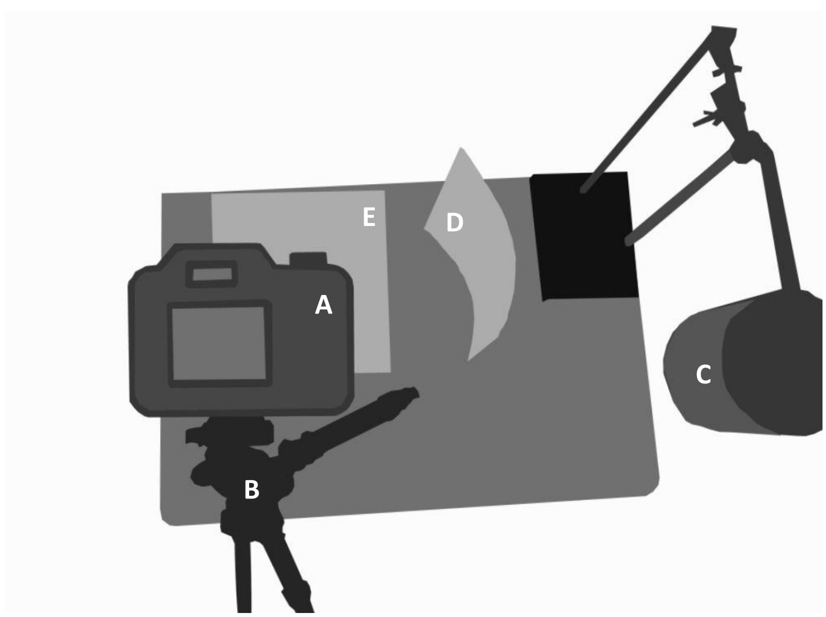

Figure 5: A bird's-eye view of the (A) camera, (B) tripod, (C) lamp, and (D&E) Teflon sheets used to photograph *L. corniculatus.*

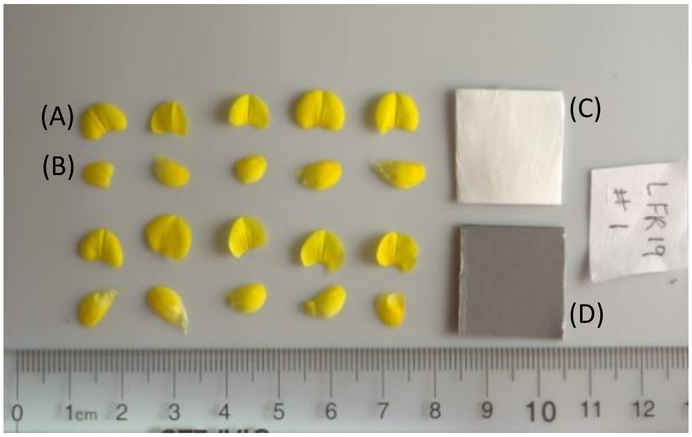

Figure 6: Visible light photo example. Standard petals (A) and wing petals (B) were arranged and photographed next to a white (C) and grey (D) standard.

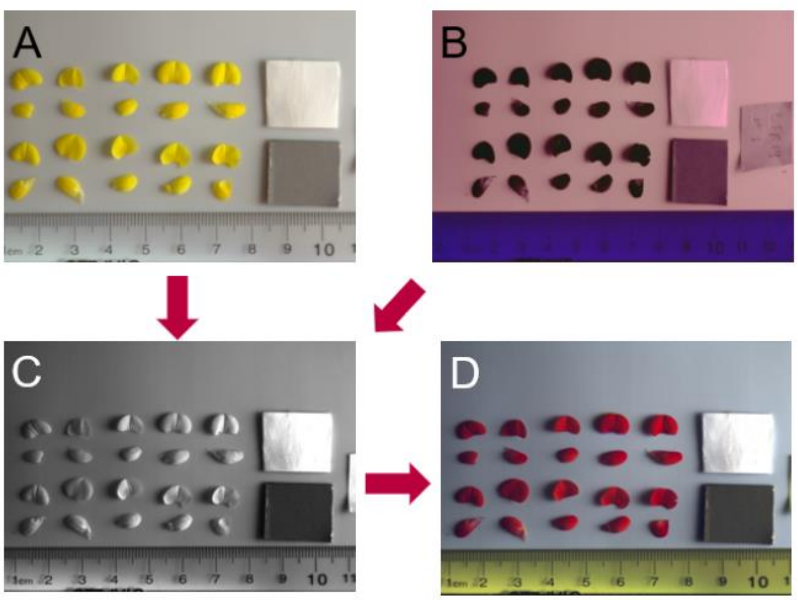

Figure 7: Image processing procedure combining (A) visible light and (B) ultraviolet light images to create a (C) multispectral image. This image was converted to a cone-catch model and (D) false-color image using the visual systems of a honey bee (Dyer *et al.* 2011).

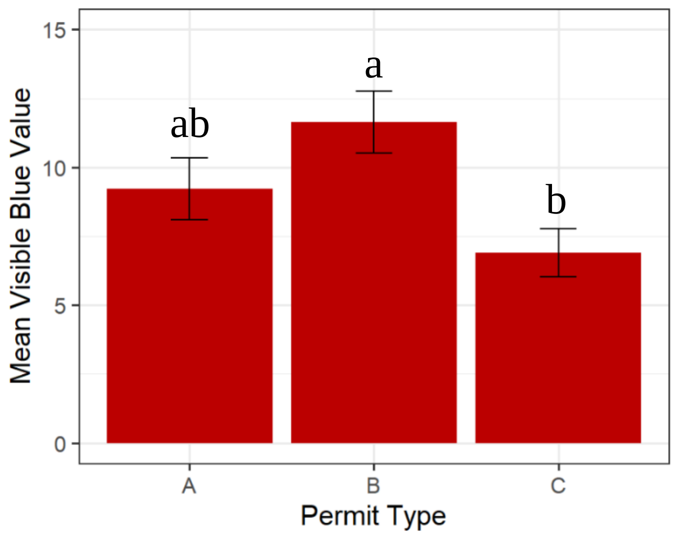

Figure 8: The average reflectance value of Visible Blue in each permit type. Different letters indicate p-values < 0.05. This relationship is similar for standard and wing petals.

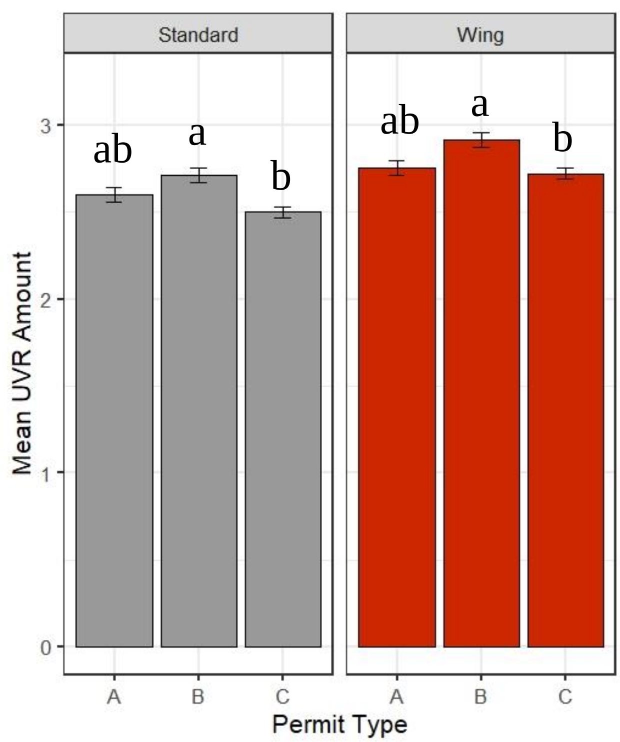

Figure 9: The average reflectance value of UV red in the wing and standard of each permit type. B and C permits were significantly different, with different significance values for standard and wing petals (p = 0.008 vs. p = 0.014).

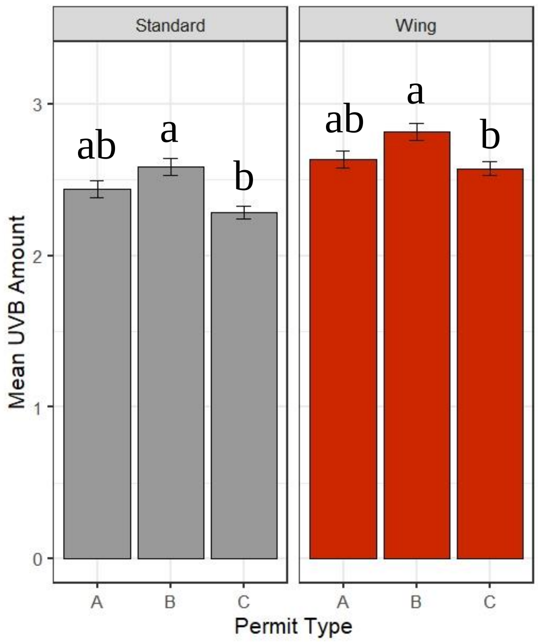

Figure 10: The average reflectance value of UV blue in the wing and standard of each permit type. B and C permits were significantly different, with different significance values for standard and wing petals ($p = 0.006$ vs. $p = 0.012$).

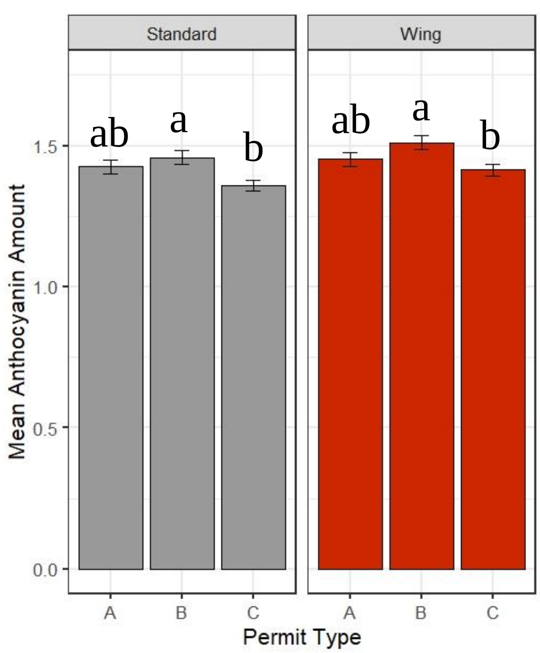

Figure 11: The average reflectance value of anthocyanin concentrations in the wing and standard of each permit type. B and C permits were significantly different, with different significance values for standard and wing petals ($p = 0.030$ vs. $p = 0.034$).

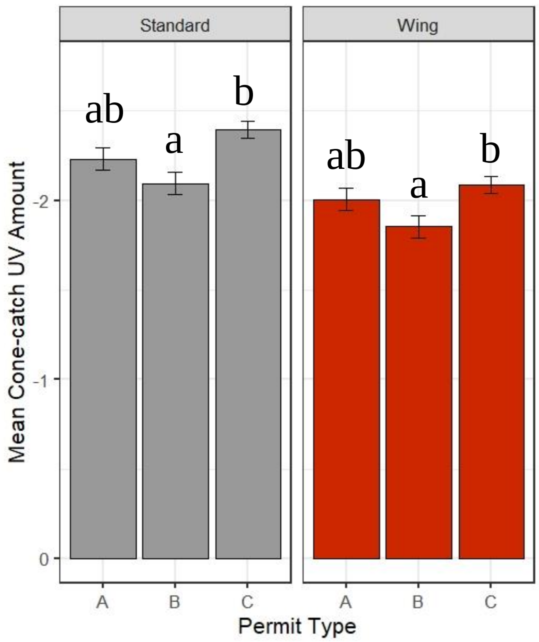

Figure 12: The average reflectance value of cone-catch UV in the wing and standard of each permit type. B and C permits were significantly different, with different significance values for standard and wing petals ($p = 0.012$ vs. $p = 0.039$).

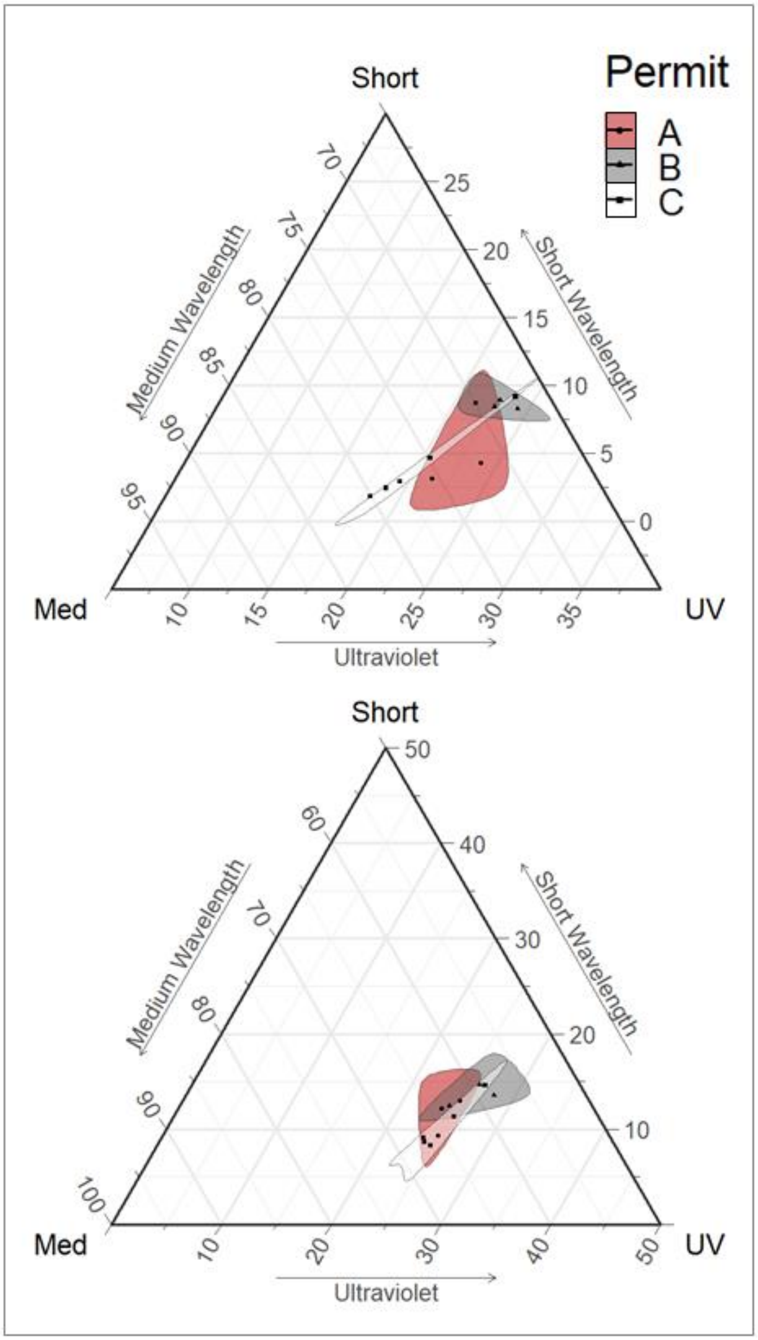

Figure 13: Flower color of L. corniculatus across permit site plotted in honey bee color space for (A) Standard and (B) Wing petals. Dots represent average color at a permit site. Colored ellipses represent different permit type, with those closer two ellipses are to each other, the more similar the groups are to each other.